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Regulation of Plant Pyruvate Dehydrogenase Complex by Phosphorylation[1]

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ABSTRACT

The ATP-dependent inactivation of the pyruvate dehydrogenase complex (PDC) was examined using ruptured mitochondria and partially purified pyruvate dehydrogenase complex isolated from broccoli and cauliflower (*Brassica oleracea*) bud mitochondria. The ATP-dependent inactivation was temperature- and pH-dependent. [^{32}P]ATP experiments show a specific transphosphorylation of the γ-PO_4 of ATP to the complex. The phosphate attached to the PDC was labile under mild alkaline but not under mild acidic conditions. The inactivated-phosphorylated PDC was not reactivated by 20 mM $MgCl_2$, dialysis, Sephadex G-25 treatment, apyrase action, or potato acid phosphatase action. However, partially purified bovine heart PDC phosphatase catalyzed the reactivation and dephosphorylation of the isolated plant PDC. The ATP-dependent inactivation-phosphorylation of the PDC was inhibited by pyruvate. It is concluded that the ATP-dependent inactivation-phosphorylation of broccoli and cauliflower mitochondrial PDC is catalyzed by a PDC kinase. It is further concluded that the PDC from broccoli and cauliflower mitochondria is capable of interconversion between an active (dephosphorylated) and an inactive (phosphorylated) form.

The pyruvate dehydrogenase multienzyme complex (PDC) catalyzes a key oxidative decarboxylation reaction:

$$CH_3COCOOH + CoA + NAD^+ \rightarrow$$
$$CH_3CO\text{-}S\text{-}CoA + CO_2 + NADH + H^+$$

The complex is composed of three enzymes operating sequentially: pyruvate dehydrogenase (decarboxylating), dihydrolipoate transacetylase, and dihydrolipoate dehydrogenase.

The PDC[2] has been purified from numerous nonplant tissues (3, 7, 16) and recently from plants (18, 19, 20). The properties of the complex from nonplant tissues have been reviewed recently by Reed *et al.* (16), Denton *et al.* (7), and Hucho (9). PDC has been shown to be regulated by product or feedback inhibition by NADH and acetyl-CoA (7, 9, 16). Regulation by other metabolites such as GTP, PEP, fructose bis-P, citrate, glyoxylate, and hydroxypyruvate have also been reported (7, 9, 16).

A second level of regulation which involves interconvertible forms (phosphorylated and dephosphorylated) of the complex has been established in mammalian tissues and *Neurospora crassa* (10, 12, 16, 25). In mammalian tissues, the phosphorylation (inactivation) of PDC is catalyzed by a specific MgATP-dependent kinase (5, 10). Conversion of the phosphorylated form of PDC to the active (dephosphorylated) complex is catalyzed by a specific Mg^{2+}-dependent phosphatase (8, 10, 11). Recently, we reported that PDC from broccoli mitochondria was inactivated by ATP by what appeared to be a similar phosphorylation mechanism (15).

No report of any posttranslational modifications of an enzyme by phosphorylation could be found in the plant literature (17, 21, 23) prior to our preliminary report of the phosphorylation of PDC from broccoli florets (15). This report describes the evidence for the existence of a PDC kinase in broccoli and cauliflower mitochondria, and further supports our initial conclusion that the plant PDC is regulated by an enzymically catalyzed phosphorylation and dephosphorylation mechanism.

MATERIALS AND METHODS

Chemicals. NAD^+, CoA, ATP, ADP, GTP, CTP, and UTP were purchased from P/L Biochemicals. Pyruvic acid, thiamine pyrophosphate, crystallized and lyophilized BSA, MES, TES, MOPS, glycylglycine, 2-mercaptoethanol, EGTA, cysteine-HCl, apyrase (grade 1), and hexokinase (crystalline suspension) were purchased from Sigma Chemical Co. Triton X-100 was obtained from Packard. [γ-^{32}P]ATP and [α-^{32}P]ATP were purchased from ICN.

Assay for Pyruvate Dehydrogenase Complex. The initial rate of the over-all PDC reaction was determined by monitoring NADH formation at 340 nm and 27 C with a Gilford model 2000 recording spectrophotometer. The standard assay mixture contained 87.5 μmol MOPS + 87.5 μmol glycylglycine (pH 8.1), 0.01 μmol thiamine pyrophosphate, 1 μmol $MgCl_2$, 2.4 μmol NAD^+, 0.12 μmol lithium CoA, 2.6 μmol cysteine-HCl, 1 μmol potassium pyruvate, and enzyme complex in a total volume of 1 ml. The final pH of the assay mixture was 7.9. The reaction was initiated with pyruvate unless otherwise indicated. A unit of enzyme activity is expressed as 1 μmol of NADH formed/min and is based on the initial rate. Specific activity is defined as units/mg of protein. Protein concentrations were determined by the method of Lowry *et al.* (13) using crystalline BSA as the standard.

Isolation of Mitochondria. Mitochondria were isolated from broccoli and cauliflower (*Brassica oleracea*) floral buds as described by Rubin and Randall (18). Cauliflower and broccoli were obtained from local markets and the outer 2 to 4 mm of the floral buds removed with a razor blade and kept at 4 C until processed. Cauliflower mitochondria were isolated from 600 g of floral buds by homogenizing 100-g batches in 200 ml of extraction medium (0.4 M sucrose, 50 mM K-phosphate [pH 7.7], and 5 mM EGTA) for 30 sec with a Polytron (PT 20-ST, speed 7). The pooled brei was filtered through four layers cheesecloth and two layers Miracloth (Chicopee Mills), and centrifuged at

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[2] Abbreviations: PDC: pyruvate dehydrogenase complex; EGTA: ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid; MOPS: morpholinopropane sulfonic acid.

400*g* for 10 min. The supernatant was centrifuged 30 min at 14,500*g* and the pellet suspended in 250 ml of extraction medium containing 20 mM 2-mercaptoethanol. This suspension was then centrifuged at 14,500*g* for 20 min, and the resulting pellet was suspended in 20 mM K-phosphate (pH 7) containing 20 mM 2-mercaptoethanol. The suspension was centrifuged at 14,500*g* for 20 min and the pellet suspended in a minimal amount of 20 mM K-phosphate (pH 7). The mitochondria were shell-frozen in a dry ice-isopropyl alcohol bath and stored at −20 C. Intact mitochondria were isolated by the same extraction, filtration, and centrifugation procedure, and stored on ice.

Crude mitochondria suspensions from broccoli were prepared by homogenizing 800 to 1000 g of floral buds in 2.5 volumes of medium (0.5 M sucrose, 0.1 M K-phosphate [pH 7.8], 0.1% BSA, 5 mM EGTA, and 13.5 mM 2-mercaptoethanol). Homogenization was perfomed in 100-g batches using a Polytron homogenizer (PT 20-ST, speed 7.5) for 30 sec. The brei was filtered through eight layers of cheesecloth and two layers of Miracloth. The pooled filtrate was centrifuged at 400*g* for 15 min. The resulting supernatant was centrifuged for 30 min at 14,500*g* to pellet the mitochondria. The mitochondrial pellet was gently resuspended in a 1:1 dilution of the extraction medium with 25 mM K-phosphate (pH 7), and recentrifuged at 12,000*g* for 30 min. The mitochondria were washed by resuspending in 250 ml of the same wash medium and repelleted as above. The final mitochondrial pellet was resuspended in a minimal amount (about 10–15 ml) of 25 mM K-phosphate (pH 7) and either shell-frozen using a dry ice-isopropyl alcohol bath or stored on ice.

Inactivation of Pyruvate Dehydrogenase Complex. ATP-dependent inactivation of the complex was performed by incubating 0.5-ml aliquots of enzyme at 25 C with 0.5 to 1 μmol of ATP. The reaction mixture was assayed just prior to ATP addition (zero time) and at various time intervals after ATP addition, to determine PDC activity. Controls (without ATP) were treated identically. When intact mitochondria were used as the enzyme source, the mitochondria were incubated with 1% Triton X-100 at 25 C for 5 min and the PDC activity determined prior to addition of ATP. The difference in activity between zero time and times after ATP addition was a measure of ATP-dependent PDC inactivation or pyruvate dehydrogenase complex "kinase" activity.

Phosphorylation of Pyruvate Dehydrogenase Activity. Incorporation of ^{32}P from [γ-^{32}P]ATP was used to show phosphorylation of the pyruvate dehydrogenase complex. The conditions for inactivation were the same as above except that the [γ-^{32}P]ATP (specific radioactivity 28,000 cpm/nmol) was placed on Whatman 3MM paper (2.5 × 2.5 cm) and dropped into 1 liter of cold 10% (w/v) trichloroacetic acid for 2 hr. The papers were washed three times with cold trichloroacetic acid, twice with ethanol, and twice with ether. The papers were dried and the ^{32}P incorporated into trichloroacetic acid-precipitable protein was measured by a liquid scintillation spectrometer. Parallel measurements to determine PDC activity were performed on the same enzyme sample and control incubations using boiled enzyme were performed to determine nonspecific binding of ^{32}P.

Bovine Heart Phosphatase. Heart PDC phosphatase was isolated from 6.8 kg of beef heart according to the method of Linn *et al.* (11). The phosphatase was concentrated by vacuum dialysis for 18 hr and stored on ice. Heart PDC phosphatase was assayed according to Hucho *et al.* (10).

RESULTS

ATP-dependent Inactivation of PDC. The PDC was released from broccoli mitochondia using 1% Triton X-100 and centrifugation at 27,000*g* for 15 min. Inactivation of solubilized PDC was time- and ATP-dependent (Fig. 1). Solubilized PDC from cauliflower mitochondria was incubated for 4 min with 1 mM ATP to partially inactivate the complex, and passed through a Sephadex G-25 column (20 × 1.5 cm) equilibrated with 50 mM MOPS, pH 7. The Sephadex G-25 treatment did not restore PDC activity, but prevented further inactivation by separating the ATP from the enzyme complex (Table I).

PDC from 1% Triton X-100-treated broccoli mitochondria was incubated with 2 mM ATP. After a 1-min and a 15-min incubation with the ATP, half of the inactivation mixture was added to 20 units of apyrase, which hydrolyzes ATP to AMP and Pi (Fig. 2A). Apyrase treatment after 1 min of ATP incubation prevented the complete inactivation of the PDC. After a 15-min incubation apyrase treatment had no effect on the inactivated PDC. The addition of hexokinase and glucose to the PDC-ATP inactivation mixture also prevented the complete inactivation of the PDC (Fig. 2B).

The PDC was isolated and highly purified from the mitochondria of broccoli and cauliflower florets by procedures previously reported (18), with the exception that dithiothreitol was omitted from the mitochondrial extraction buffer. Omission of the sulfhydryl protecting agent was necessitated by the

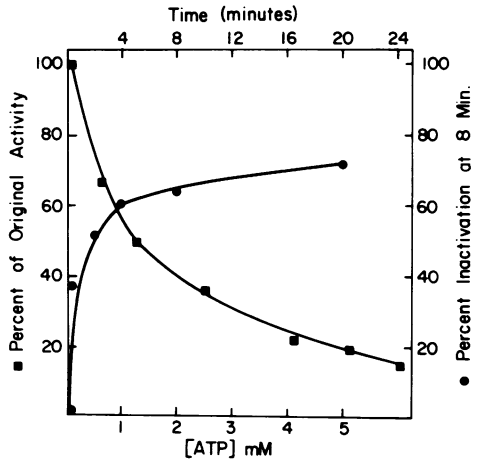

FIG. 1. Time and concentration dependency of the ATP inactivation of PDC isolated from Triton X-100-lysed broccoli mitochondria. The inactivation mixture contained 0.25 ml broccoli mitochondria (PDC, sp. act. 0.03), 50 μl 10% Triton X-100, 0.10 ml H_2O, and either no ATP or 1 μmol ATP (■) incubated for various periods of time or with various concentrations of ATP incubated for 8 min (●).

Table I

ATP Dependent Inactivation of the PDC from Cauliflower Mitochondria

Isolated cauiflower mitochondria were lysed with 1% Triton X-100 and centrifuged at 27,000 x g for 15 min to remove the membrane debris. The supernatant containing the PDC activity was incubated with 2 mM ATP for the indicated times, whereupon 20 μl aliquots were withdrawn and assayed. The control was identical without ATP present. After 4 min of incubation with ATP, a 0.5 ml sample was layered onto a G-25 Sephadex column equilibrated with 50 mM MOPS, pH 7.0. The PDC activity and protein concentration from the peak tubes were then determined.

EXPERIMENTAL CONDITIONS	INCUBATION TIME	ACTIVITY REMAINING	SPECIFIC ACTIVITY
	MIN	%	μmol NADH / min·mg protein
Control	0	100[a]	0.110
	10	100	0.110
+2mM ATP	4	20	0.022
	6	5	0.006
+2mM ATP +G-25 Sephadex	4	21	0.023

[a] 100% PDC activity represents 0.77 units of activity per ml.

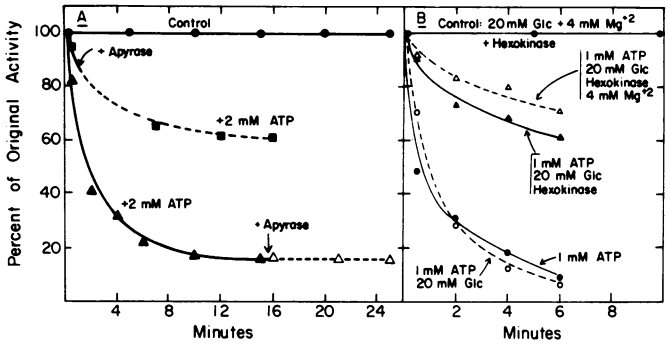

FIG. 2. Inactivation of PDC from Triton X-100-lysed cauliflower and broccoli mitochondria by ATP. A: Inactivation mixture contained 0.25 ml broccoli mitochondria (PDC, sp. act. 0.03); 50 μl 10% Triton X-100; 0.10 ml H_2O; and either (●) no ATP addition, (■) 1 μmol ATP with 20 units apyrase added after 30 sec, (▲) 1 μmol ATP with 20 units apyrase added after 15 min (△). The final volume of the incubation mixture was 0.5 ml. B: Inactivation mixture contained 0.25 ml cauliflower mitochondria (sp. act. 0.04); 50 μl 10% Triton X-100; 0.15 ml H_2O; and either (△) 0.5 μmol ATP, 10 μmol glucose, 2 μmol $MgCl_2$, and 10 units hexokinase; (▲) 0.5 μmol ATP, 10 μmol glucose, and 10 units hexokinase; (●) 0.5 μmol ATP; or (○) 0.5 μmol ATP, and 10 μmol glucose, all in a final volume of 0.50 ml.

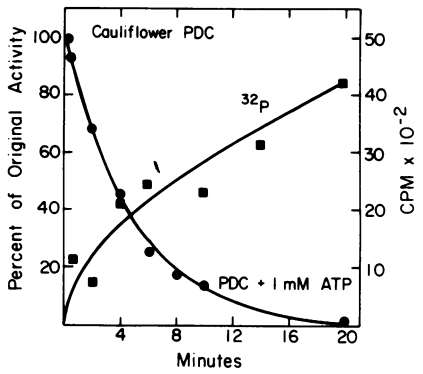

FIG. 3. Concomitant inactivation of cauliflower. PDC with γ-^{32}P incorporation from γ-[^{32}P]ATP. The incubation mixture contained 10 μmol TES (pH 7.5), 0.25 ml cauliflower PDC (1.15 mg protein, sp. act. 1.6); and (●) 0.5 μmol ATP; (■) 0.50 μmol γ-[^{32}P]ATP (specific radioactivity 28,000 cpm/nmol).

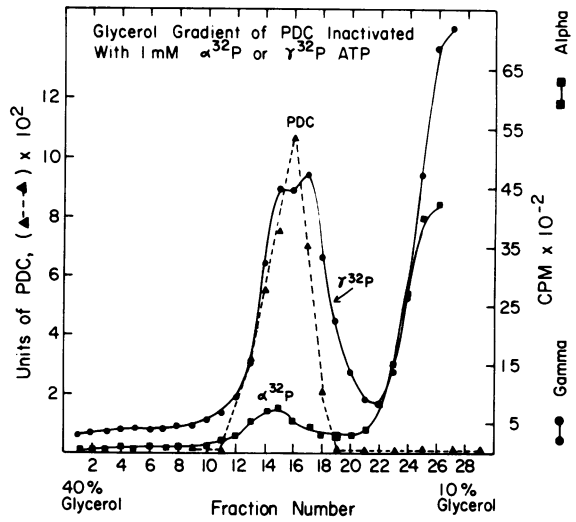

FIG. 4. Glycerol gradient. Pyruvate dehydrogenase complex was purified from broccoli mitochondria and the incubation mixture contained 0.5 ml PDC, (3 mg protein, sp. act. 1.3), 15 μmol TES (pH 7.5), 0.8 μmol $MgCl_2$, and 2 μmol ATP containing either (●) γ-[^{32}P]ATP (specific radioactivity 28,000 cpm/nmol) or (■) α-[^{32}P]ATP (specific radioactivity 28,000 cpm/nmol) in a final volume of 1 ml. The inactivated PDC was layered on a 10 to 40% (v/v) 17-ml glycerol gradient, centrifuged at 82,500g for 9.5 hr and fractionated into 0.5-ml fractions. The fractions were counted for ^{32}P incorporation and assayed for PDC activity (▲).

apparent lability of the PDC kinase activity in the presence of these reagents. The PDC activity was lower in the absence of these reagents. After the PEG fractionation, specific activities of 1.3 (broccoli) and 1.6 (cauliflower) were observed, as compared to approximately 2.5 (broccoli) and 3.2 (cauliflower) in the presence of dithiothreitol.

Purified cauliflower PDC was 90% inactivated after 10 min in the presence of 1 mM ATP (Fig. 3). This inactivation of the PDC by ATP was paralleled by the incorporation of ^{32}P from γ-[^{32}P]ATP into trichloroacetic acid-precipitable protein. No inhibition was observed over this time period when ATP was omitted from the incubation mixture.

To ensure that the trichloroacetic acid-precipitable ^{32}P counts (from the [γ-^{32}P]ATP inactivation) represented actual phosphorylation of complex, the cauliflower PDC was purified through the PEG fractionation (step III, sp. act. 1.6) as previously described (18). The PDC was inactivated by incubating for 15 min with either 2 mM [γ-^{32}P]ATP (28,000 cpm/nmol) or 2 mM [α-^{32}P]ATP (28,000 cpm/nmol). The PDC was then centrifuged through 9 ml of 10% glycerol at 271,900g for 2 hr. The enzyme complex was resuspended in 25 mM K-phosphate (pH 7) and layered on 17-ml 10 to 40% (v/v) linear gylcerol gradients. The gradients were centrifuged at 82,500g for 9.5 hr in a SW 27.1 rotor. Figure 4 shows the combined profile of the gradients for the inactivated PDC and a control gradient of nonphosphorylated PDC which was treated identically. The γ-^{32}P peak is coincident with the PDC peak indicating a specific phosphorylation. The only major α-^{32}P activity was in the portion of the gradient lacking PDC.

To determine the nature of the phosphate linkage to the PDC, purified cauliflower PDC was inactivated with [γ-^{32}P]ATP and then incubated for 18 hr in either 0.25 N NaOH or HCl. As shown in Table II, the trichloroacetic acid-precipitable counts were labile in alkali but not in acid, indicating that the ^{32}P was esterified to the PDC.

Temperature and pH Dependence of the PDC Phosphorylation. The rate of the ATP-dependent inactivation of PDC increased with temperature. Figure 5 shows the inactivation of PDC from Triton X-100-lysed broccoli mitochondria with 2 mM ATP at 24 C compared to the inactivation of 8 C, followed by warming to 24 C. A similar temperature dependency was found with partially purified cauliflower PDC, where there was approximately a 40% inactivation after 10 min of incubation (2 mM ATP) at 4 C and a 70% inactivation at 25 C.

The ATP-dependent inactivation of PDC was also pH-dependent. The inactivation of both cauliflower and broccoli PDC proceeded most rapidly at pH 7.5, but was only slightly less at pH 8.5 and 6.5, indicating a fairly broad pH range of inactivation (Fig. 6). Above pH 8.5 the PDC was unstable and the activity was quickly lost.

Reactivation of the Phosphorylated PDC. The addition of high concentrations of $MgCl_2$ (10–20 mM) to the inactivated, phosphorylated form of the PDC has been shown to reactivate and dephosphorylate mammalian and *N. crassa* complex (8, 10, 12, 22, 25). The reactivation resulted from the presence of a native Mg^{2+}-dependent PDC phosphatase. Attempts to reactivate the inactivated PDC from either broccoli or cauliflower by addition of 10 to 20 mM $MgCl_2$ and 1 to 5 mM $CaCl_2$ to stimulate a native phosphatase were unsuccessful. As previously mentioned, passage of the inactivated PDC through a Sephadex G-25 column did not reactivate the complex nor did apyrase treatment (Table I and Fig. 2B). Table III shows that efforts to reactivate inactivated PDC using either potato acid phospha-

Table II

Mild Alkali and Acid Treatment of Phosphorylated PDC

PDC purified from cauliflower mitochondria (sp. act. 1.6) was inactivated using 2 mM γ-^{32}P (28,000 cpm/nmole). Aliquots, 50 µl, were removed at the indicated time and placed on Whatman 3 MM filter paper, and the ^{32}P incorporated into trichloroacetic acid precipitable protein was determined. The inactivated complex was then incubated with either 0.25 N NaOH or HCl for 18 hr, and the incorporated ^{32}P determined. A control incubation using boiled PDC was treated similarly to determine nonspecific ^{32}P binding. The values shown are corrected by this control (1520 cpm).

EXPERIMENTAL TREATMENT	INCUBATION TIME	NET CPM
PDC plus	0.5 min	2026
+2mM γ-^{32}P ATP	2	3415
+0.25 N NaOH	18 hr	350
+0.25 N HCl	18 hr	3928

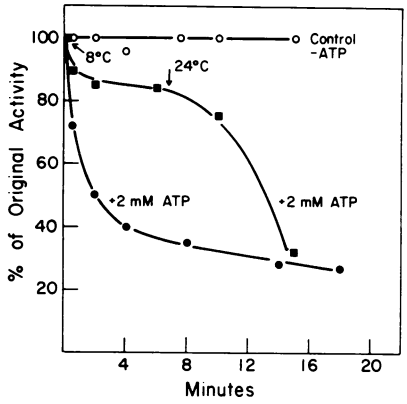

FIG. 5. Effect of temperature on the ATP-dependent PDC inactivation. PDC isolated from broccoli mitochondria were lysed with 1% Triton X-100 and centrifuged at 27,000g for 15 min to obtain the solubilized PDC (sp. act. 0.08). The PDC was then incubated with 1 µmol ATP in a total volume of 0.50 ml either at 8 C or 24 C (■) for the indicated times. Controls with ATP (●) and without ATP (○) were incubated at 24 C.

tase and/or dialysis treatments were also unsuccessful. However, the addition of partially purified heart PDC phosphatase to inactivated broccoli PDC resulted in a 45% reactivation in about 45 min (Fig. 7B). The heart PDC phosphatase catalyzed the release of ^{32}P from phosphorylated broccoli PDC.

Nucleotide Specificity of Inactivation. The nucleotide specificity of the PDC kinase reaction was examined using 2 mM ATP, CTP, GTP, and UTP. The effects of the various nucleotides are shown in Figure 8. After 12 min of incubation, the PDC from Triton X-100-lysed broccoli mitochondria was inactivated 70% with ATP, 52% with UTP, 30% with CTP, and no inactivation was observed with GTP. The effect of ADP on the inactivation of the PDC from either Triton X-100-lysed broccoli or cauliflower mitochondria is shown in Figure 9, A and B, along with the inactivation observed with 2 mM ATP after 10 min (Fig. 9A). With the cauliflower complex, 2 mM ADP was 72% as effective as 1 mM ATP after 6 min of incubation. AMP and Pi were without effect on the PDC activity (Fig. 9).

The ADP-dependent inactivation of PDC was further investigated by adding hexokinase and glucose to the inactivation mixture (Fig. 9C). The inactivation of PDC was reduced by over 70% after 6 min of incubation and indicated that the ADP-dependent inactivation of PDC was mediated through ATP. To determine if the ATP might be generated by a mitochondrial adenylate kinase, partially purified cauliflower PDC (sp. act. 1.6) was incubated with 2 mM ATP or ADP (Fig. 9D). The partially purified PDC was inactivated 78% by ATP after a 10-

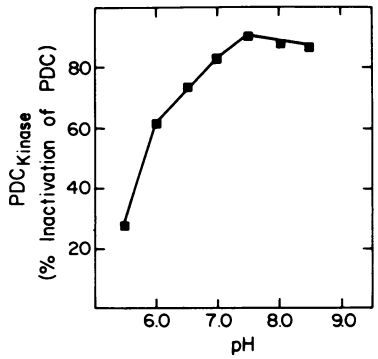

FIG. 6. Effect of pH on the ATP-dependent inactivation of cauliflower PDC. PDC was solubilized from cauliflower mitochondria using 1% Triton X-100. The incubation mixture contained 0.19 ml PDC (sp. act. 0.1), 5 µmol MES, 5 µmol MOPS, 5 µmol glycylglycine, and 0.5 µmol ATP in a final volume of 0.25 ml. Points shown are pH values of final reaction mixtures.

Table III

Reactivation of ATP Inactivated Broccoli PDC

Broccoli PDC was purified to a specific activity of 1.3. PDC was inactivated with 1 mM ATP for 15 minutes, and 10 units of potato acid phosphatase were then added in the experiments indicated.

Dialysis Time	PDC -ATP -Phosphatase	PDC -ATP +Phosphatase	PDC +ATP -Phosphatase	PDC +ATP +Phosphatase
0	10.5[a] (100)[b]	10.5 (100)	2.8 (27)	2.8 (27)
3 hr	9.8 (93)	9.0 (86)	2.0 (19) +phosphatase	1.5 (14)
6 hr	-	-	1.9 (18)	-

[a] units of PDC activity

[b] Percent of original activity given in parentheses.

min incubation and only 30% with ADP. The purified complex was inactivated 6% by 2 mM CTP and no inactivation was found with UTP (data not shown).

Examination of the ADP by hexokinase, glucose, and glucose-6-P dehydrogenase indicated 1.5% ATP contamination in the ADP. This contamination was confirmed by paper chromatography of the nucleotides in an isobutyric acid-NH_4OH-H_2O (66:1:33) solvent system.

Pyruvate Inhibition of PDC Phosphorylation. The effect of pyruvate on the ATP-dependent inactivation of partially purified broccoli and cauliflower PDC was examined. Inactivation of PDC by 2 mM ATP was inhibited by pyruvate at four different concentrations as shown in Figure 10. PDC inactivation was completely inhibited at 1 mM pyruvate, and a 50% inhibition of phosphorylation was observed at 0.1 mM pyruvate after 8 min of incubation. Similar results were observed with the cauliflower complex.

DISCUSSION

The data reported here indicate that the PDC activity in broccoli and cauliflower mitochondria can be regulated by phosphorylation-dephosphorylation similar to mammalian and *Neurospora* systems (7, 16, 25). The concomitant inactivation of crude or purified PDC with incorporation of ^{32}P from γ-[^{32}P]ATP into trichloroacetic acid-precipitable protein is indicative of a PDC kinase (12). The inactivation of PDC by ATP was temperature-, pH-, and concentration-dependent (Figs. 1, 5, 6), further supporting our conclusion of an enzyme-catalyzed (PDC kinase) inactivation. The ATP inactivation of the PDC by [γ-^{32}P]ATP *versus* [α-^{32}P]ATP (Fig. 4) shows an enzymic

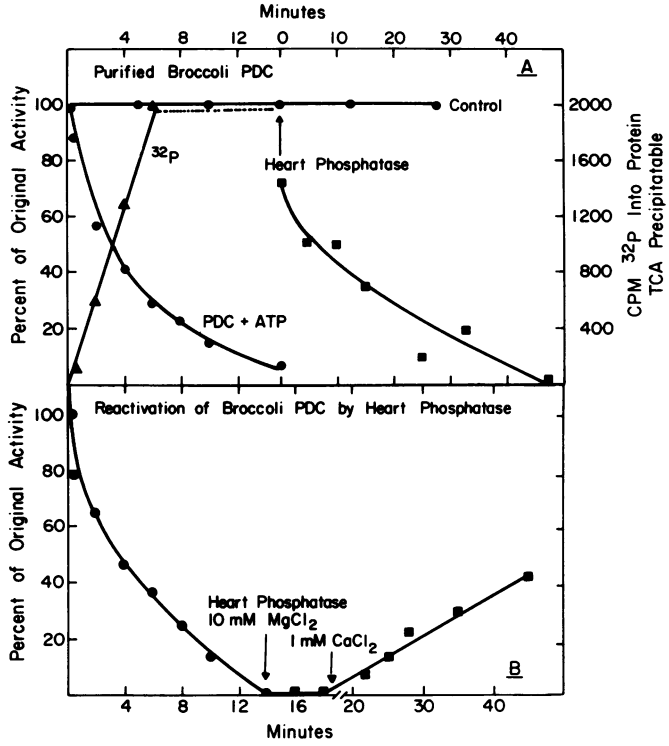

FIG. 7. Effect of heart PDC phosphatase on inactivated PDC from broccoli. PDC was purified from broccoli mitochondria as previously described (18). A: Complex inactivated by addition of 1 mM ATP (●); 1 mM ATP containing γ-[^{32}P]ATP (specific radioactivity 28,000 cpm/nmol), was treated at the indicated times with 10 mM $MgCl_2$, 1 mM $CaCl_2$, and 100 μl crude heart PDC phosphatase (▲,■). B: Experiments were performed as above with addition of (●) 2 mM ATP followed by (■) 10 mM $MgCl_2$, 1 mM $CaCl_2$, 100 μl heart PDC phosphatase.

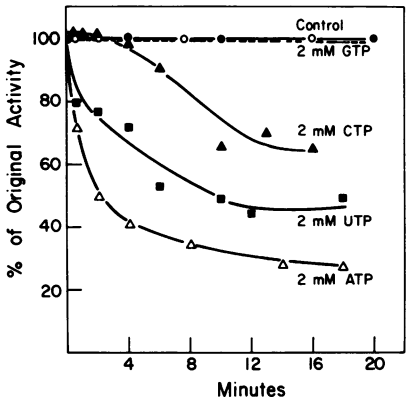

FIG. 8. Effect of nucleotide triphosphates on the inactivation of PDC. PDC (sp. act. 0.10) from broccoli mitochondria lysed with 1% Trition X-100 was incubated with 2 mM nucleotides. Additions: (○) none; (●) GTP; (▲) CTP; (■) UTP; (△) ATP.

transphosphorylation of the γ-phosphate of the ATP to the PDC. Further support for the transphosphorylation of the PDC is the fact that the ^{32}P incorporated into trichloroacetic acid-precipitable protein (PDC) was alkali- but not acid-labile (Table II). This is indicative of an ester linkage between the ^{32}P and PDC. Inactivation of plant PDC by noncovalent binding of the ATP, ADP, AMP, or Pi was considered unlikely since apyrase treatment (Fig. 2), passage of the inactivated enzyme through Sephadex G-25 (Table I), or dialysis (Table III) of the inactivated PDC were all ineffective in reactivating the enzyme. However, apyrase action and Sephadex G-25 chromatography (Fig. 2 and Table I) could curtail complete inactivation of the PDC, if the treatments were performed prior to full inactivation of the complex by ATP.

The inactivation and phosphorylation process was quite specific for ATP, even though the PDC activity in crude mitochondrial extracts could be inactivated by UTP, CTP, and ADP

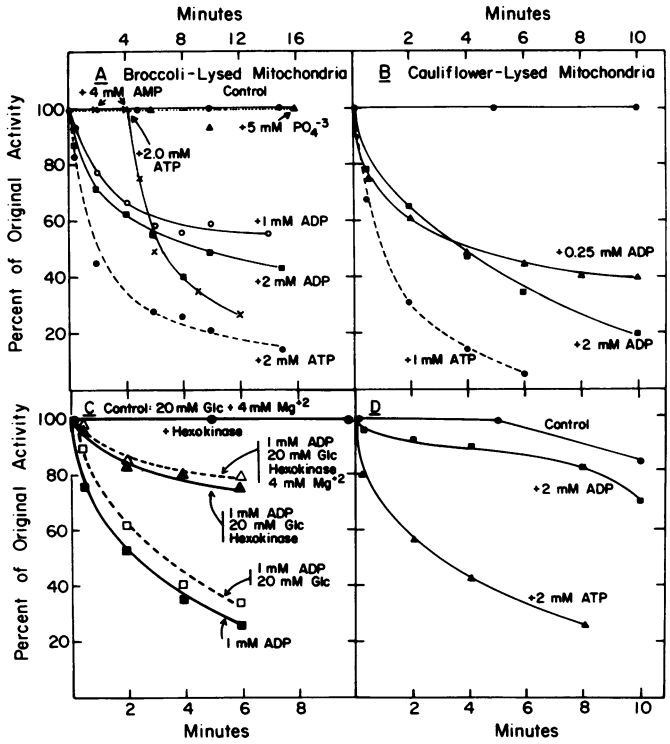

FIG. 9. Effect of ADP on the PDC from cauliflower and broccoli mitochondria. A: Incubation mixture contained 0.25 ml broccoli mitochondria (sp. act. 0.07), 50 μl 10% Triton X-100, and 0.10 H_2O. Additions were as described in the figure in a final volume of 0.50 ml. B: Incubation mixture contained 0.25 ml cauliflower mitochondria (sp. act. 0.032), 50 μl 10% Triton X-100, and 0.10 ml H_2O. Additions were as described in the figure in a final volume of 0.50 ml. C: Incubation mixture contained 0.25 ml cauliflower (sp. act. 0.032), 50 μl 10% Triton X-100, and 0.10 ml H_2O. Additions were as described in the figure in a final volume of 0.5 ml. Ten units hexokinase were used in those assays where indicated. D: Incubation mixture contained 125 μl PDC (sp. act. 1.6), 5 μmol TES (pH 7.5). Additions were (●) none; (■) 0.5 μmol ADP; (▲) 0.5 μmol ATP in a final volume of 0.25 ml.

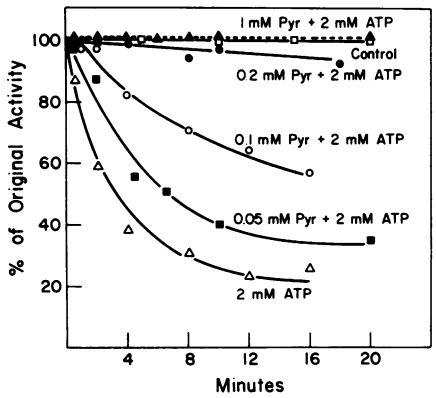

FIG. 10. Effect of pyruvate on the ATP-dependent inhibition of broccoli PDC. PDC was solubilized from Triton X-100-lysed mitochondria as previously described in Figure 5. Incubation mixture contained 2 mM ATP and pyruvate at 1 mM (▲); 0.2 mM (●); 0.1 mM (○); 0.05 mM (■); 0 (△).

(Figs. 8 and 9). We conclude that the ability of UTP, CTP, and ADP to inactivate the complex is the result of the presence of nucleotide diphosphate kinase and adenylate kinase with the unpurified enzyme which could generate ATP. This conclusion is supported by the inability of these nucleotides to inactivate purified PDC, along with the finding that the addition of hexokinase and glucose was able to prevent the inactivation in the absence of ATP.

Reactivation and dephosphorylation of the PDC-phosphate in mammalian and *Neurospora* systems can be accomplished by simply increasing the $MgCl_2$ concentration to about 10 mM (10, 12, 25), which activates the specific PDC phosphatase. The presence of native PDC phosphatase, using either inactivated broccoli or cauliflower complex, was not detectable by increasing the Mg^{2+}, Mn^{2+}, or Ca^{2+} levels. Potato acid phosphatase will dephosphorylate several phosphoproteins including phosphorylase *a* (1). However, as shown in Table III, the potato acid phosphatase would not reactivate the phosphorylated plant PDC. Bovine heart PDC phosphatase catalyzed the dephosphorylation and reactivation (Fig. 7) of the phosphorylated-inactive plant PDC. This fact supports our conclusion that the phosphorylation-inactivation process is specific and that a phosphorylation-dephosphorylation mechanism of regulation can occur with the plant PDC. We believe that the apparent lack of a native plant PDC phosphatase is due to lability or loss of the enzyme under the isolation conditions employed. The mammalian PDC phosphatase is known to be loosely associated with the complex and is frequently removed by ultracentrifugation of the complex (11). We also found that the broccoli or cauliflower PDC kinase activity has a rather labile nature, especially when the mitochondria or the PDC are isolated in the presence of thiols. This is the opposite of what is observed with heart PDC kinase (11) which is activated by thiols.

The pyruvate inhibition of the inactivation-phosphorylation of the PDC (Fig. 10) appears to be noncompetitive *versus* ATP (data not shown) and is similar to that observed with the mammalian PDC kinase (10). The effect of pyruvate on the ATP inactivation of PDC also adds support to our conclusion for the presence of a PDC kinase and a phosphorylation-dephosphorylation control for plant PDC.

The picture that is emerging from our studies of the plant PDC is that of a highly regulated enzyme complex. The plant PDC is regulated through product feedback inhibition by NADH and acetyl-CoA (6, 18, 20), and possibly by metabolites such as hydroxypyruvate and glyoxylate (8, 18). A second level of control, that of a phosphorylation-dephosphorylation cycle also appears to be functioning. Inactivation of the PDC appears to be carried out by a Mg-ATP-dependent kinase similar to the enzyme described from various mammalian tissues (4, 17). The PDC kinase activity would be a response to the total energy state of the cell (mitochondrion), and when the ATP levels increase, the kinase inactivates the PDC. The phosphorylation-inactivation process is apparently also controlled by the pyruvate (substrate of PDC) inhibition of the PDC kinase. Assuming that there is a PDC phosphatase operating *in vivo*, the pyruvate effect would be to shift the PDC to the active side of the phosphorylation-dephosphorylation balance. We are currently investigating the regulation of the kinase and the PDC phosphatase *in vivo*. However, with the details thus far described it appears that the plant PDC is a highly regulated enzyme and will play a key role in regulating carbon flow between glycolysis, the Krebs cycle, fatty acid metabolism, and the unique metabolic events in photosynthetic tissue (2, 14, 24). The role that the PDC plays in regulating the carbon flow through these various pathways is now under examination.

In this report we have presented the evidence for the existence of a posttranslational modification of an enzyme by a phosphorylation mechanism. This is the first description of such a mechanism for a higher plant system (21, 23). The existence of this important mechanism for the regulation of the plant PDC extends this type of control into the higher plant kingdom and suggests the probability of finding other enzymes controlled by a similar mechanism.

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